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Chip-based amperometric enzyme sensor system for monitoring of bioprocesses by flow-injection analysis

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ABSTRACT

A microfluidic chip integrating amperometric enzyme sensors for the detection of glucose, glutamate and glutamine in cell-culture fermentation processes has been developed. The enzymes glucose oxidase, glutamate oxidase and glutaminase were immobilized by means of cross-linking with glutaraldehyde on platinum thin-film electrodes integrated within a microfluidic channel. The biosensor chip was coupled to a flow-injection analysis system for electrochemical characterization of the sensors. The sensors have been characterized in terms of sensitivity, linear working range and detection limit. The sensitivity evaluated from the respective peak areas was 1.47, 3.68 and 0.28 μAs/mM for the glucose, glutamate and glutamine sensor, respectively. The calibration curves were linear up to a concentration of 20 mM glucose and glutamine and up to 10 mM for glutamate. The lower detection limit amounted to be 0.05 mM for the glucose and glutamate sensor, respectively, and 0.1 mM for the glutamine sensor. Experiments in cell-culture medium have demonstrated a good correlation between the glutamate, glutamine and glucose concentrations measured with the chip-based biosensors in a differential-mode and the commercially available instrumentation. The obtained results demonstrate the feasibility of the realized microfluidic biosensor chip for monitoring of bioprocesses.

1. Introduction

Stringent monitoring and control of bioprocess parameters is crucial for process optimization in terms of high-quality products with a sufficiently high yield. Various technological approaches for instrumentation of bioprocess monitoring are established. Besides expensive and labor-intensive chromatographic analysis methods, optical sensors (Höpfner et al., 2010; Lam and Kostov, 2010; Marose et al., 1999) as well as electrochemical (bio)sensors (Locher et al., 1992; Sonnleitner, 2000) or a combination of both (Akin et al., 2011) find widespread application. In view of the large number and diversity of possible process parameters, knowledge of the concentration levels of essential nutrients such as glutamine and glucose is of particular interest (Duba and Preuß, 2010; Schneider and Grolms, 2011). The amperometric oxidation of hydrogen peroxide as product of a (bi)enzymatic conversion of these substrates remains the most common approach for analytical detection and quantification and is routinely performed in biochemistry analyzers. However, the need for trained people on the one hand and the frequent replacement of the sensor membranes on the other hand results in an expensive and time-consuming analysis. In this context, miniaturized silicon-based sensors have the potential to reduce the costs of analysis (Bäcker et al., 2011b; Krommenhoek et al., 2007).

In this work, a Si-based biosensor chip with an integrated microfluidic channel for simultaneous amperometric detection of three analytes, namely glucose, glutamate and glutamine is presented. For this, glucose oxidase (GOD), glutamate oxidase (GLOD) and a two-enzyme system made up of glutaminase (GLMN) and GLOD has been immobilized onto patterned platinum thin-film electrodes by means of cross-linking with glutaraldehyde. A linear microfluidic channel was realized on the chip by means of SU-8 technology. For electrochemical characterization, the biosensor chip was coupled to a flow-injection analysis (FIA) system. The developed biosensor chip has been tested for the simultaneous detection of glucose, glutamine and glutamate in a batch hybridoma cell-culture medium and compared with reference analytical methods. The possibility of reducing the intrinsic cross-sensitivity of the glutamine sensor towards glutamate by using differential-mode measurements for improved glutamine detection will be discussed.

2. Materials and methods

Glutamate, glutamine, glutaraldehyde, bovine serum albumin (BSA) and the buffer components were purchased from

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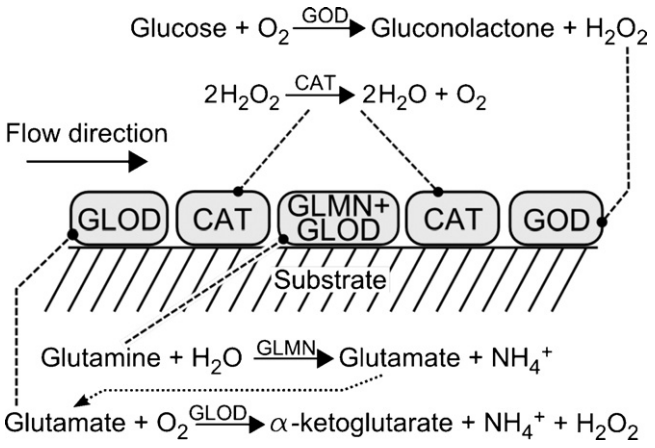

Fig. 1. Cross-sectional scheme of the biosensor chip illustrating the position of the enzyme membranes and their corresponding working principle, respectively.

Sigma-Aldrich Co. (St. Louis, MO, USA). The enzymes used were glucose oxidase (EC 1.1.3.4) from *Aspergillus niger*, L-glutamate oxidase (EC 1.4.3.11) from *Streptomyces* sp. (recombinant, expressed in *E. coli*, 9.2 U/mg), glutaminase (EC 3.5.1.2) from *E. coli* (78 U/mg), catalase (CAT, EC. 1.11.1.6) from bovine liver and were purchased from Sigma-Aldrich, too.

2.1. Chip processing and enzyme immobilization

The sensor chip was fabricated by means of conventional silicon technology. To passivate the Si substrate, 500 nm SiO_2 was grown by thermal wet oxidation of a p-Si wafer. Electron-beam evaporation was used to deposit on the SiO_2 surface 20 nm titanium as adhesion layer and subsequently, a 200 nm thick platinum layer as electrode material for amperometric detection. Then, the metal layers were patterned by means of photolithography and lift-off technique. For the integration of the biosensor chip into the FIA system, a flow channel was realized on the chip. This flow channel was formed by photolithographic structuring of a 100 μm thick SU-8 photoresist (Micro resist technology GmbH, Berlin, Germany) layer. The width of the channel was 1.75 mm, the distance from inlet to outlet amounted to be 15 mm. Finally, the processed structures were separated into chips with sizes of 15 mm × 20 mm, cleaned and glued onto printed circuit boards. Electrical connection was provided by means of an ultrasonic wedge bonder. The enzyme-based biosensors were constructed by coupling an enzyme membrane with Pt electrodes. The immobilized GOD membrane was prepared by the following method: first, GOD was dissolved in phosphate buffer resulting in an enzyme concentration of 670 U/ml. Next, this aqueous solution was mixed with BSA (10 wt%) and glutaraldehyde (2.5 vol%) in glycerin (10 vol%). The volumetric ratio of the three components of this enzyme solution was 1–2–2 (enzyme–BSA–glutaraldehyde). For preparation of the glutamate sensor, 0.1 mg GLOD was dissolved in 10 μl BSA solution. One part of this aqueous solution was mixed with glutaraldehyde forming the glutamate sensor membrane. Another part was mixed with 10 U of GLMN dissolved in citrate buffer (approximately 1 U/μl). After thorough mixing, glutaraldehyde was added to promote cross-linking of the ingredients. Aliquots of 1.5 μl of the corresponding enzyme solution were then dropped on the platinum electrode resulting in an enzyme loading of approximately 0.1–0.2 U/electrode. Fig. 1 depicts a cross-sectional scheme of the biosensor chip illustrating the working principles of the individual sensors. The functioning of the glutamine biosensor is based on a two-step enzymatic reaction: first glutamine is converted to glutamate and NH_4^+ by the GLMN; during the second enzymatic reaction, GLOD converts glutamate to α-ketoglutarate, NH_4^+ and H_2O_2. The glutamine sensor was positioned downstream to the glutamate sensor. In this way, the glutamate content of the sample is already reduced to some extent by the glutamate sensor and causes less interference with the glutamine sensor. The glucose sensor was positioned closest to the outlet. To minimize the effect of cross-talk between the sensors, additional membranes containing approximately 1000 U CAT for decomposition of hydrogen peroxide in the channel were immobilized in between the glutamate and the glutamine sensor membrane and the glutamine and glucose sensor membrane, respectively, via cross-linking, too.

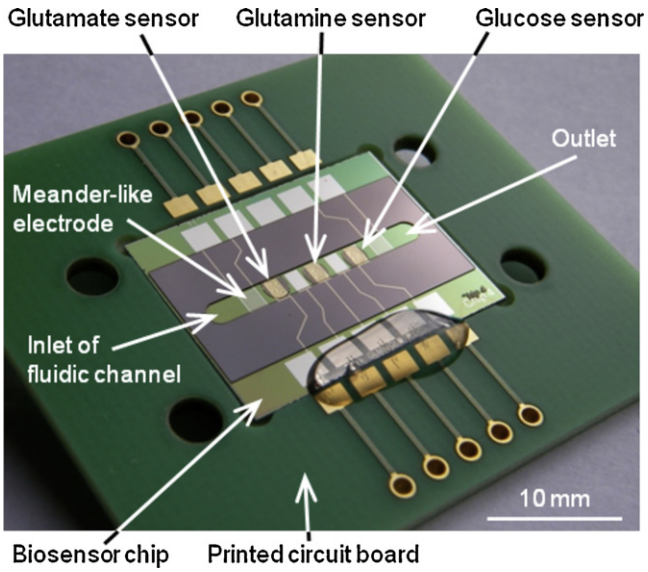

Fig. 2. Photograph of the biosensor chip with the integrated microfluidic channel and an array of amperometric enzyme sensors for the simultaneous detection of glucose, glutamate and glutamine. The additional meander-like electrodes can serve as temperature sensor.

Finally, the chip was rinsed with buffer solution to remove unbound components and stored at 4 °C until required. Fig. 2 shows a photo of the biosensor chip with the integrated microfluidic channel and an array of amperometric enzyme sensors for the simultaneous detection of glucose, glutamate and glutamine.

2.2. Electrochemical sensor characterization

For the electrochemical characterization the electrodes were connected to a potentiostat (PalmSens with multichannel multiplexer, Palm Instruments BV, Netherlands). A conventional liquid-junction Ag/AgCl electrode (Metrohm) was used as a reference electrode. To oxidize the hydrogen peroxide that is produced during the enzymatic reactions, a constant potential of +600 mV vs. Ag/AgCl was applied to the platinum working electrodes. Using the amperometric detection method, the steady-state current usually serves as a measure for the substrate concentration. By application of a FIA system, a larger number of parameters can be utilized for data analysis, e.g. the peak height, the peak area or the peak width. In this way, the quality of the data can be improved (Becker et al., 2007). Furthermore, the usage of the peak area of the Faradaic current as an integral parameter minimizes errors due to spikes of the signal. Additionally, the working range of the sensor can be increased, since saturation of the enzymes as a limiting factor for the maximum current is reduced as a result of sample dispersion (Rehbock et al., 2008). Hence, the data evaluation of the experiments was performed based on the peak area. For the measurements, the microfluidic channel was covered with a perspex coverplate having holes for inlet and outlet tubes for connection

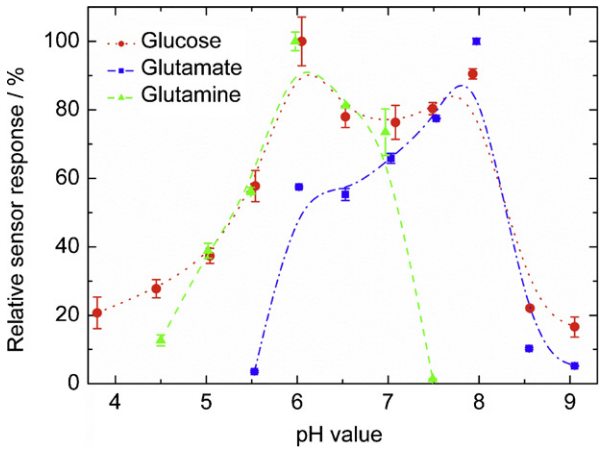

Fig. 3. Relative sensor response of the glucose (red dots), glutamate (blue squares) and glutamine (green triangles) sensor to threefold injections of 1 mM substrate in dependence of the pH value.

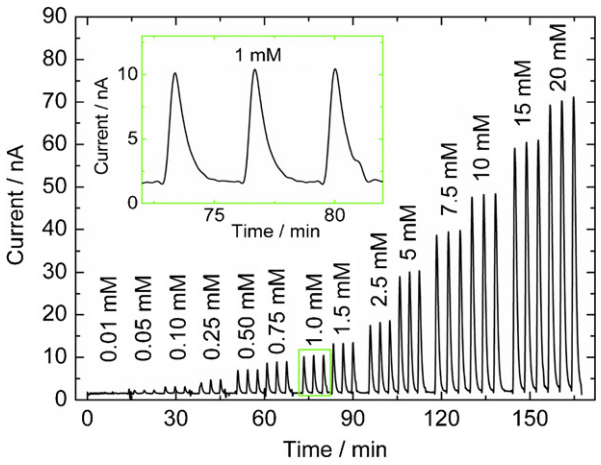

Fig. 4. Flow-injection response of the glutamine biosensor in the concentration range from 0.01 mM to 20 mM glutamine. The inset exemplarily magnifies the response to injections of 1 mM glutamine.

to the FIA system (FIAlab-2500, FIAlab Instruments Inc., USA). The volume of the injection loop was set to 500 μl; the flow rate was 1.3 ml/min. All measurements were conducted at ambient temperature.

3. Results and discussion

3.1. Effect of pH on the response of the biosensors

The effect of pH on the response of the individual biosensors was studied using buffer solutions with different pH values in the range from pH 4 to pH 9. The buffer solutions used were 100 mM citrate buffer for pH < pH 6, 100 mM phosphate buffer for pH 6 ≤ pH ≤ pH 8 and 100 mM Tris buffer for pH > pH 8. The pH-dependent response of the glucose, glutamate and glutamine sensors is depicted in Fig. 3. The presented data points are an average of threefold injections of 1 mM substrate, the error bars represent ±1 standard deviation. For each sensor, the highest response was taken as 100% and all other values were normalized to this value.

The pH dependency of the glucose sensor followed a bell-shaped course. The strongest sensor response was found for the citrate buffer with pH 6. A broad pH range with a sensor response of 80% or higher was obtained between pH 6 and pH 8. Even for the most extreme pH values of solutions used in this study (pH 4 and pH 9), no total loss of enzyme activity but only a reduction to approximately 20% was observed. In case of the glutamate sensor, a distinct maximum signal for the glutamate sensor was found at pH 8. This value is higher than reported by White et al. (1994) (pH 7) and Tian et al. (2009) (pH 7.4) but within the range of up to pH 8.5 as described by Villarta et al. (1992). For pH values below pH 6, the sensor response was significantly reduced. No evaluable sensor signals were obtained in buffer solutions with a pH below pH 5.5. Both the glucose and the glutamate sensor showed a sharp peak (decrease in relative sensor response) when the pH was larger than pH 8. In comparison to the glutamate sensor, the response of the glutamine sensor was narrowed and shifted towards lower pH values due to the GLMN contributing to the reaction, which has an activity optimum at pH 5 (Schügerl et al., 1991). The maximum sensor response was achieved in a solution with pH 6. This optimum pH is slightly above values found in literature (pH 5.5) (Villarta et al., 1992).

In general, for an optimal sensor performance each of the biosensors should be supplied with an individual carrier solution with the above formulated pH optima. However, this requires a more complex experimental setup in terms of the FIA system as well as the complicated design and fabrication of the microfluidic chip, because every sensor has to be addressable with an independent fluid line. On the other hand, deviations from the pH optimum might be tolerable, if the sensor response is only affected to some extent. Therefore, some compromise should be found. Since the main interest is the monitoring of the glutamine and glucose concentration, the operation at pH 6 would be beneficial. Considering the pH optimum of the glucose sensor, this compromise does not limit the sensor performance. In case of the glutamate sensor, the sensor response is reduced by approximately 40% in comparison to its optimum value at pH 8. Based on the results and discussion presented, further characterization of the biosensors has been done in solutions with pH 6.

3.2. Electrochemical characterization of the biosensor chip

The biosensors were then systematically characterized with respect to their detection limit, working range and sensitivity towards the specific analytes. For this, samples containing the corresponding substrate in the concentration range from 0.01 mM to 20 mM were injected in the fluidic channel. Each concentration level was measured three times. As an example, Fig. 4 depicts the response of the glutamine sensor. The inset in the plot shows a magnified view of the sensor responding to three glutamine injections of 1 mM revealing the typical peak formation as a result of probe dispersion and the good reproducibility of the sensor signal.

In Fig. 5, the calibration curves for the three biosensors are presented in a double-logarithmic scaling. The presented data points correspond to the averaged peak area of threefold injections of the respective substrate. Under the present flow rate and injection volume, the calibration curves were linear up to 20 mM for glucose and glutamine and up to 10 mM for glutamate. The sensitivity was 1.47 μAs/mM (R = 0.998) for the glucose sensor, 3.68 μAs/mM (R = 0.999) for the glutamate sensor and 0.28 μAs/mM (R = 0.999) for the glutamine sensor. No sensor saturation was observed in the concentration range investigated. The lower detection limit amounted to be 0.05 mM for the glucose and glutamate sensor, respectively, and 0.1 mM for the glutamine sensor. In a previous study, the long-term characteristics of the sensors were investigated separately, indicating a decrease in enzyme activity after nine days of approximately 40% in case of the glutamate sensor and 85% in case of the glutamine sensor, respectively (Bäcker et al., 2011a).

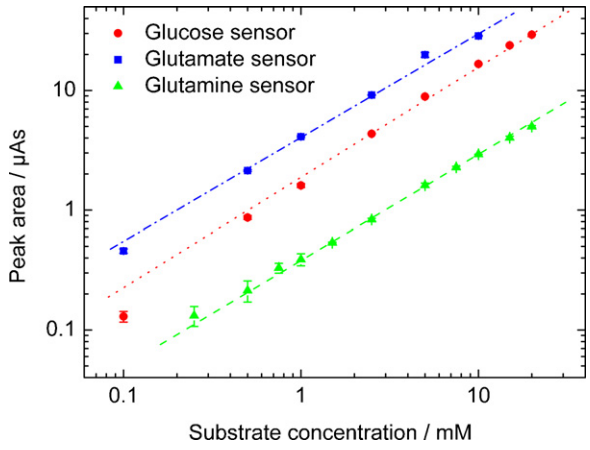

Fig. 5. Calibration curves of the glucose (red dots), glutamate (blue squares) and glutamine (green triangles) sensor obtained by threefold injections of the respective substrate of different concentration; error bars indicate the standard deviation.

3.3. Simultaneous measurements of all biosensors

All three biosensors studied in this work are based on the same detection principle, i.e., the oxidation of hydrogen peroxide produced by the enzymatic reactions. In the chosen FIA setup, due to the transportation of the hydrogen peroxide in the microfluidic channel by diffusion and the fluid flow itself, there is a risk of cross-talk between the sensors. For reduction of this effect, CAT membranes were immobilized (upstream) in between the sensor membranes to decompose the hydrogen peroxide to water and oxygen. To investigate whether cross-talk occurred, simultaneous measurements with all three sensors connected and polarized to the same potential were performed. Fig. 6 exemplarily depicts the response of the three sensors to *glutamate* (Fig. 6a) and *glutamine* (Fig. 6b) injections in the concentration range from 0.1 mM to 10 mM. As can be seen in Fig. 6a, an increasing peak height with increasing substrate concentration was observed for the glutamate sensor. At the same time, the intrinsic sensitivity of the glutamine sensor towards glutamate becomes apparent. This response of the glutamine sensor to glutamate was even higher than to glutamine at the same substrate concentration levels (see Fig. 6b). A minor signal peak from the glucose sensor was observed, which can be attributed to incomplete decomposition of hydrogen peroxide released from the upstream positioned glutamine and glutamate sensors. In case of glutamine injections, neither the glucose nor the glutamate sensor (but only the glutamine sensor) showed a distinct sensor response. However, due to the bi-enzymatic reaction, the sensor signal of the glutamine sensor (Fig. 6b) was tenfold lower than for the glutamate sensor (Fig. 6a). When injecting glucose of various concentrations, similar results were obtained (data not shown). Here, only the glucose biosensor delivered a sensor current, while the upstream positioned glutamine and glutamate sensors have not been affected by the injected glucose.

3.4. Determination of substrate concentration in real samples

The knowledge of the glucose and glutamine concentrations as essential nutrients for the supply of the cells is of particular importance for bioprocess control. Therefore, further experiments were performed to investigate the ability of the biosensor chip for determination of glucose and glutamine in different samples from real fermentation broths. Beside glucose, such samples typically contain both, glutamine and glutamate. The glutamine sensor detects the hydrogen peroxide which is a byproduct of the conversion of glutamate catalyzed by GLOD. This glutamate can have two origins. It can be the product of the GLMN reaction or it is endogenously present in the sample. Thus, the presence of glutamate in the sample hampers the glutamine analysis and the response of the glutamine sensor (i.e., the peak area) consists of two portions. The first portion reflects the glutamine content of the sample and the second portion reflects the glutamate content of the sample. To diminish the impact of the latter one, a differential measurement procedure was established. The procedure involves the determination of the glutamate portion to the signal of the glutamine sensor. This portion is then subtracted from the overall peak area. For evaluation of this differential measurement procedure, samples were drawn over a period of three days from a batch cultivation of hybridoma cells. The measurement was performed in citrate buffer (pH 6) with two injections per sample. Prior to the analysis of the fermentation samples, the three biosensors were calibrated in solutions of known concentration of corresponding substrates. Additionally, the glutamine sensor was calibrated in glutamate solutions. The glutamate sensor was applied to quantify the glutamate concentration of the present sample. With the knowledge of the glutamate concentration, its contribution to the peak of the glutamine sensor can be estimated by means of the additional calibration measurement of the glutamine sensor in glutamate solutions. This allows the correction of the response of the glutamine sensor for the glutamate content and, subsequently, the determination of the glutamine concentration of the sample. The calculated values for the concentration of glucose, glutamate and glutamine as measured by the biosensor chip were compared to reference analyzers. In case of the glucose sensor, the Eppendorf EBIO compact was used, for analysis of glutamate and glutamine the YSI 2700 analyzer served as a reference system (Fig. 7).

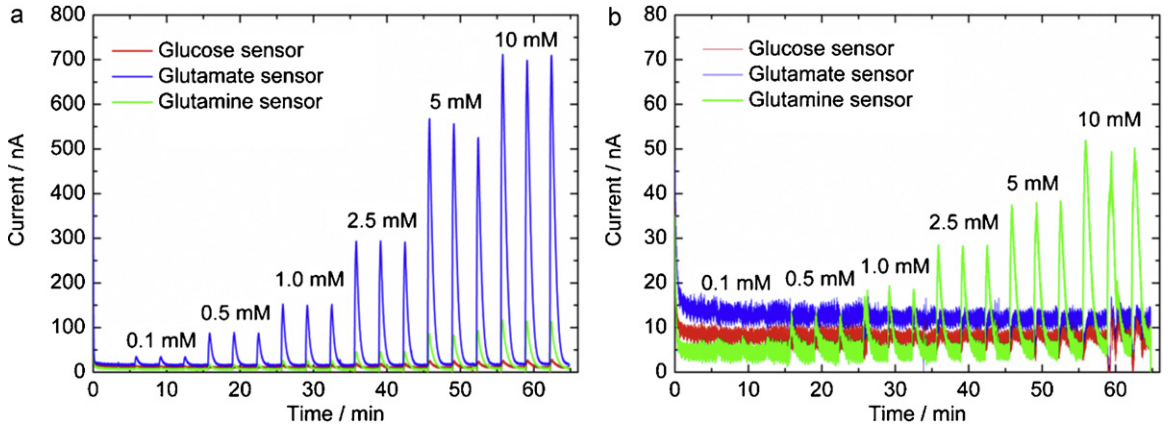

Fig. 6. Simultaneous measurements with all three biosensors in response to *glutamate* (left) and *glutamine* (right) injections recorded in citrate buffer pH 6.

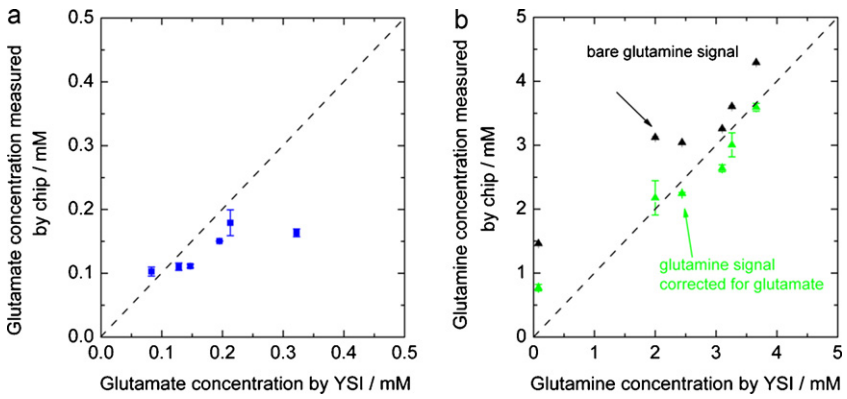

Fig. 7. Comparison between the glutamate data (a) and glutamine data (b) obtained by the biosensor chip and the reference system. The contribution of glutamate to the signal of the glutamine sensor (black triangles) was subtracted (green triangles).

In Fig. 7, the results obtained with the glutamate and glutamine sensor are compared with reference data recorded by the YSI 2700 analyzer. Fig. 7a contains the data for glutamate detection. A good correlation between the data measured by the chip and the reference system was achieved. For the sample with highest glutamate concentration the value obtained by the biosensor chip deviated by approximately 0.1 mM to lower values. Fig. 7b shows the data for glutamine detection. To illustrate the improvement of using the differential measurement, both the data of the bare glutamine sensor signal (black triangles) as well as the data which was corrected for the glutamate contribution (green triangles) is depicted. Without application of the differential measurement, the calculated glutamine concentrations were systematically higher than the reference measurement. The contribution of the glutamate in the sample resulted in an error of up to ≈50%. The application of the differential method (green triangles) significantly improved the agreement between the data obtained by the chip and the reference system.

The results of the glucose sensor in comparison to the reference system are plotted in Fig. 8. Again, the data from the biosensor chip correlated well with the reference system. However, the data obtained by the biosensor chip showed a trend to quantify the glucose concentration slightly lower than the reference system.

4. Conclusions and outlook

A silicon-based microfluidic biosensor chip for the simultaneous determination of glucose, glutamate and glutamine has been developed. The preliminary experiments successfully demonstrate the feasibility of the realized biosensor chip for monitoring the nutrient concentration in fermentation processes. An optimization of the FIA parameters (flow rate, sample volume, etc.) could further improve the performance of the sensor chip. The use of silicon technology for the production of the sensor chip in conjunction with a straightforward immobilization approach could result in a cost-effective and small-sized analysis tool. Moreover, it provides the opportunity to integrate additional sensors for the detection and control of further parameters, for instance, platinum thin-film structures for temperature or impedimetric measurements (Poghossian and Schöning, 2004; Bäcker et al., 2009) or silicon-based pH sensors (Schöning et al., 2005).

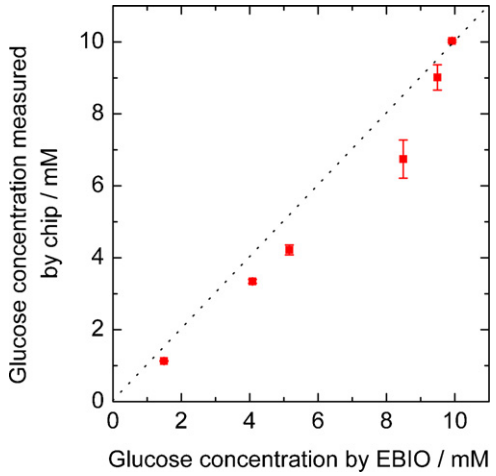

Fig. 8. Comparison between the glucose data obtained by the biosensor chip and the reference system.

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